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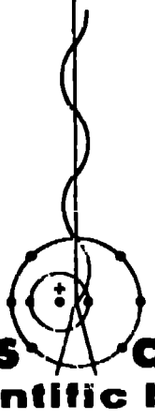

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Cell-Cycle-Related Biosynthesis

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In 1963, Mazia proposed a speculative model in which the structure of chromatin was envisioned to exist in a state of continuous flux throughout the entire mammalian cell cycle, interphase as well as mitosis. Among a variety of possibilities, the notion of a dynamic "chromosome cycle" superimposed on the division cycle was attractive since it provided one potential avenue for modulating control of cell-cycle events. That is, chromatin structure might dictate in part the types of physical and chemical interactions allowable within the genome at any given stage of the cell cycle (e.g., accessibility of DNA polymerases to initiation sites during S phase, etc.). Furthermore, the state of chromatin might affect the nature of interactions between DNA and chemotherapeutic agents which depend upon covalent bond formation or intercalation for activity.

With these considerations in mind, the state of chromatin during the cell cycle was examined utilizing synchronized cultures of line CHO Chinese hamster cells. Results described in this report support Mazia's dynamic chromosome cycle model (Mazia 1963) and further indicate that DNA-interactive chemotherapeutic agents elicit different types of kinetic responses in treated cells, perhaps suggesting a degree of specificity of interaction between various alkylating and intercalating agents and the genome.

CELL-CYCLE-SPECIFIC CHANGES IN CHROMATIN ORGANIZATION: STUDIES WITH SARKOSYL CRYSTALS

If the structure of chromatin does indeed vary throughout interphase and mitosis, then chemical probes which interact with components of chromatin should register quantitative differences in reactivity at various stages of the cell cycle. Conversely, a static chromatin structure should result in a uniform degree of reactivity throughout the entire cell cycle. To distinguish between these possibilities, a series of studies was initiated in which "sarkosyl" crystals were employed to probe the organization of chromatin in synchronized populations of CHO cells. The crystals formed by the interaction of sodium lauroyl sarcosinate with $MgCl_2$ may be utilized to measure specific associations between DNA and lipid-protein complexes in whole cells or nuclei (Tremblay et al. 1969; Hanoaka and Yamada 1971; Hildebrand and Tobey 1973). Biochemically, these complexes may be radioactively labeled with thymidine, choline, or amino acids. They are resistant to ribonuclease but sensitive to treatment with deoxyribonuclease, Pronase, sodium dodecyl sulfate, or temperatures in excess of 37°C. Less than 10% of the DNA in these sarkosyl complexes is attributable to non-specific entrapment (Hildebrand and Tobey 1973).

Once it was established that the crystal complexes were measuring specific biochemical interactions, the next step was to quantitate the amount of complex-bound DNA available for interaction in sarkosyl crystals at varying stages in the cell cycle. Cells prelabeled with ^{14}C-thymidine were resuspended in label-free

medium following mitotic selection and, at varying times after synchronization, the amount of old DNA associated with the crystals was determined for whole cells or nuclei (Hildebrand and Tobey 1973). The results (Figure 1A) indicate that a small amount of DNA was complexed to lipid-protein fragments even during mitosis and early G_1. However, the amount of DNA in sarkosyl crystals increased dramatically during G_1, commencing 2 hours prior to entry into S phase. Figure 1

Because of a naturally occurring loss of synchrony, the following procedure was employed to study events in late interphase. Cells with prelabeled DNA were first synchronized by mitotic selection, then as the cells were traversing G_1, hydroxyurea was added, yielding a population resynchronized near the G_1/S boundary. Following resuspension in drug-free medium, the cells traversed S and G_2 in a highly synchronous fashion. The results in Figure 1B indicate that the amount of DNA associated with the sarkosyl crystals was initially at a high level, then increased sharply when the cells were released from hydroxyurea blockade and allowed to traverse late interphase (Hildebrand and Tobey 1973). The level of DNA in sarkosyl crystals began to drop during late interphase and fell off dramatically at about the time the cells began dividing. The data in Figure 1 suggest a non-static association of DNA with lipophilic nuclear structures and support the notion of a dynamic "chromosome cycle."

CELL-CYCLE-SPECIFIC CHANGES IN CHROMATIN ORGANIZATION: STUDIES WITH HEPARIN

To provide an independent measure of chromatin structural changes during the cell cycle, a study was initiated (Hildebrand and Tobey 1975) with the natural polyanion, heparin, an agent which interacts predominantly with the histone component of chromatin (Arnold et al. 1972). Treatment of isolated nuclei with heparin causes histones to be removed from chromatin in a well-defined sequence, with lysine-rich histone f1 released preferentially (Hildebrand et al. 1975). Concomitant with the heparin-mediated removal of histones, the DNA is released in a highly decondensed state (Arnold et al. 1972; Berlowitz et al. 1972; Hildebrand et al. 1975). Therefore, changes in the amount of heparin required to release a given amount of decondensed DNA in a specified time period presumably reflect alterations in the organization of chromatin.

Cell-cycle studies were initiated with cultures of cells with prelabeled DNA which were synchronized by mitotic selection (Hildebrand and Tobey 1975). As the cells were traversing early interphase, aliquots were removed, and the concentration of heparin required to release 50% of the DNA during a five-minute incubation period was determined (Figure 2A). As the cells progressed through G_1 into S phase, the resistance to heparin-mediated release of decondensed DNA increased steadily (i.e., progressively greater quantities of heparin were required to achieve release of 50% of the DNA).

Figure 2

This resistance continued increasing through mid- and late-interphase in populations synchronized first by mitotic selection, then resynchronized with hydroxyurea (Figure 2B). At the time the cells began dividing, the level of heparin required to release the DNA dropped precipitously. Thus, experiments with two different probes yield the same general conclusion: the organization of chromatin varies at different stages of the cell cycle.

VARIABILITY OF ARREST SITE IN POPULATIONS TREATED WITH CHEMOTHERAPEUTIC AGENTS WHICH INDUCE CELL ACCUMULATION IN G_2

A number of chemical agents which interact with DNA can induce virtually the entire complement of cells in susceptible populations to arrest in G_2 (Bhuyan 1970; Shirakawa and Frei 1970; Barranco and Humphrey 1971; Tobey 1975; Tobey et al. 1975). Since many of these agents presumably rely upon alkylation or intercalation for activity, one might expect cells treated with such agents to respond in a generally similar fashion, implying a relatively non-specific type of interaction between the agents and the genome. In regard to kinetic response, one might predict a single point of arrest within G_2 for a variety of such agents in the event of a non-specific interaction. [Note that a variety of nutritional stresses induces cultured cells to arrest at a single locale within G_1, a site designated the "restriction point" by Pardee and associates (Pardee et al. 1974)]. Alternatively, the observation of a spectrum of unique arresting points in G_2 might imply a degree of specificity of interaction between individual agents and nuclear DNA.

Techniques of cell-cycle analysis may be utilized to reveal the precise stage within the division cycle at which progression capacity is lost in cells treated with inhibitory agents. In exponentially growing cultures treated with inhibitors, the time between addition of drug and change in division rate determines the time in the cell cycle preceding division -- the M/G_1 boundary -- at which the agent inhibited progression. Cells at a stage beyond this temporal marker at the time of drug addition continue on through the cell cycle and divide, while cells at earlier stages are prevented from progressing at the normal rate. The time is calculated back from M/G_1, since cell division is the measured parameter.

Figure 3 presents kinetic data obtained in cultures treated with two different chemotherapeutic agents: neocarzinostatin (Tobey 1975) and adriamycin (Tobey et al. 1976). In the culture treated with high levels of neocarzinostatin (Figure 3A), the cells continued dividing at the experimental division rate for 60 minutes following drug addition; thereafter, the cell number did not increase, and there was even an indication of a decrease in number of cells in the culture receiving the highest drug concentration. Lower drug concentrations incompletely inhibited cell division, but the rate of division abruptly decreased commencing at 60 minutes after addition of drug. Although the rate of progression for affected cells was dependent on the amount of drug added, the time interval between addition of drug and alteration in division rate was concentration-independent over a wide range of levels of neocarzinostatin. Thus, the terminal point of action for neocarzinostatin in CHO cells is located 60 minutes ahead of the M/G_1 boundary. Figure 3

In Figure 3B are the results obtained when cultures of exponentially growing cells were treated with adriamycin (Tobey et al. 1976). The terminal point of action of adriamycin in CHO cells is located 1.85 ± 0.1 hours ahead of the M/G_1 boundary.

In similar fashion, the terminal point of action was determined for a variety of agents which cause cells to accumulate at least transitorially in G_2, along with values for inhibitors of protein synthesis, oxidative phosphorylation, and a metaphase-arrest-inducing compound (Figure 4) (Tobey et al. 1969; Tobey 1975). It is readily apparent that the alkylating and intercalating agents tested caused the cells to stop in different portions of G_2 rather than at a single stage. The multiple arrest points support the notion of variability of response, suggesting a degree of specificity of interaction between agent and DNA.

IMPLICATIONS

Results from a variety of laboratories including our own lend support to the concept of a dynamic chromosome cycle extending throughout interphase and mitosis (Mazia 1963). For example, studies utilizing chemical probes such as actinomycin and deoxyribonuclease (Pederson 1972; Pederson and Robbins 1972), sarkosyl-Mg^{++} crystals (Yamada and Hanoaka 1973; Hildebrand and Tobey 1973), heparin (Hildebrand and Tobey 1975; Hildebrand et al. 1975), and ethidium bromide (Nicolini et al. 1975) all indicate a variable state of organization of chromatin as cells traverse the cell cycle.

What effects do these changes in chromatin structure have on interactions between alkylating/intercalating agents and DNA? Are different regions of the genome available for interaction with these agents at various stages of the cell cycle? While available data do not provide unequivocal answers to these questions, several types of experiments perhaps bear on the problems raised. If the various agents studied in this report had all reacted with the DNA in a similar fashion, one would have expected similar types of interactions, yielding a common response (i.e., arrest of cells at a common point within G_2, etc.). Obviously, the variability in stage of G_2-arrest observed in these studies (summarized in Figure 4) argues against such a simplistic notion; instead, a degree of specificity of interaction is suggested. The observed variability in response could depend on a multitude of factors, including differences in primary structure of the agents employed and the state of the chromatin at the time of exposure to drug. Figure 4

Another line of evidence suggesting variability of response (and presumably variability of interaction as well) is derived from studies of cell-cycle-specific cytotoxicity as a result of treatment of synchronized populations with DNA-interactive agents. Addition of nitrosourea derivatives or intercalating agents to synchronized cultures yields survival values with five- to ten-fold differences in survival capacity between the most sensitive and least sensitive stages of the cell cycle (cf., Barranco and Humphrey 1971; Bhuyan et al. 1972; Kim and Kim 1972; Drewinko et al. 1973; Barranco et al. 1973).

The variability in survival is probably not attributable merely to differences in the total amount of agent bound to the entire genome at different stages of the cell cycle. In this regard, studies with synchronized mouse L cells by Walker (1966) revealed that, even though cells in G_2 were approximately ten times more resistant to the lethal effects of the alkylating agent sulfur mustard than cells in S phase, nevertheless, the total amount of mustard bound per unit of DNA was identical in the two populations.

We suggest the speculative possibility that part of the variation may be attributable to the configuration of chromatin during the period of exposure to a DNA-interactive agent. That is, access of such agents to sites within the genome producing lethality may vary throughout the cell cycle. As a corollary to the above, differences in the organization of chromatin in cycling and arrested cell populations (cf., Pederson 1972; Hill and Baserga 1974) could play an important role in the efficacy of therapeutic regimens involving DNA-interactive agents.

Finally, a question arises concerning possible biochemical mechanisms which might bring about alterations in chromatin structure during the cell cycle. Several lines of evidence suggest that reversible phosphorylation of histones represents a major avenue for modulating chromatin/genome structure (Allfrey 1971; Bradbury and Crane-Robinson 1971; Adler et al. 1971, 1972). Our own studies (Gurley et al. 1974, 1975; Hohmann et al. 1975) demonstrate that, during progression through interphase and

mitosis and coincident with chromatin organizational changes, histones are phosphorylated in a highly ordered manner, exhibiting specificity for position in the cell cycle as well as specificity for both the amino acid residues and the region of the molecule phosphorylated. Figure 5 presents a partial summary of biochemical events in the CHO cell cycle and illustrates the coordination of histone phosphorylation with changes in chromatin organization. Figure 5

In view of our findings and similar studies by Marks et al. (1973) and Bradbury and associates (1973, 1974), we consider the orderly progression of specific histone modifications to play an essential role in the structure and function of chromatin as cells progress through the cell cycle. The interrelationship between these two processes possibly may one day provide a target for chemotherapy.

ACKNOWLEDGMENT

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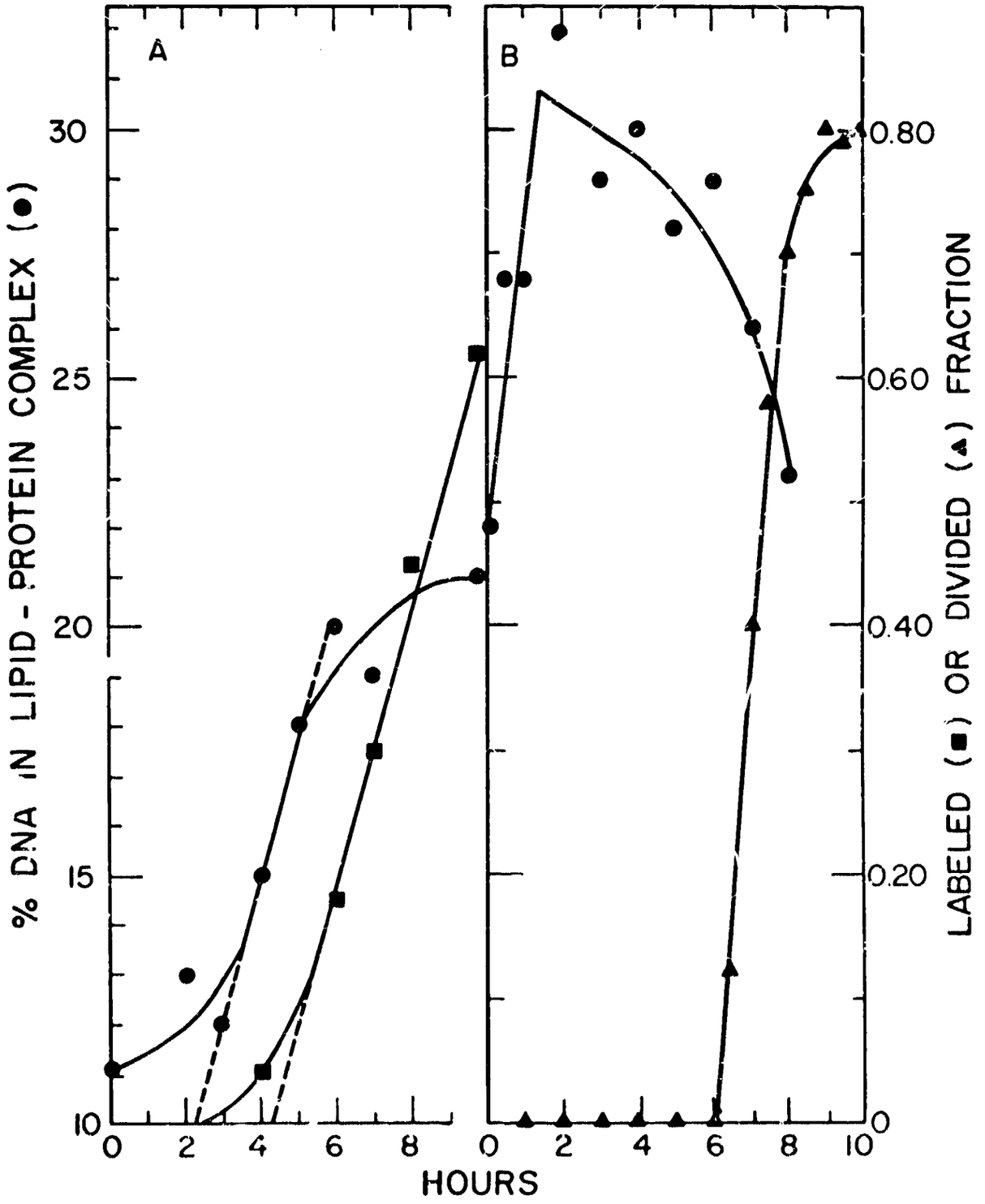
Figure 1. DNA-lipid-protein complexes in synchronized CHO cells. A, Cells were maintained for 34 hours in medium containing 0.006 μCi/ml of ^{14}C-thymidine (to label the DNA) prior to mitotic selection (Tobey et al. 1967); then these synchronized cells were resuspended in label-free medium in spinner culture. Aliquots were removed at varying times thereafter, and the amount of old, prelabeled DNA associated with lipid-protein in sarkosyl crystals was determined as described previously (Hildebrand and Tobey 1973). In addition, the fraction of cells incorporating ^{3}H-thymidine into DNA during a 15-minute pulse at 2 μCi/ml was determined via autoradiography in a non-prelabeled culture synchronized in parallel. B, Cells with DNA prelabeled as above were synchronized by mitotic selection, then resuspended in fresh label-free medium. One hour later hydroxyurea was added (final concentration 10^{-3} M), and the cells were incubated an additional nine hours. Then the cells were washed and resuspended in drug-free medium. Samples were removed at intervals thereafter to determine the amount of DNA in the sarkosyl crystals and the concentration of cells. Circles represent the sarkosyl-bound DNA, squares represent the fraction of cells labeled with ^{3}H-thymidine (i.e., fraction of cells in S phase), and diamonds represent the divided fraction ($N/N_0 - 1$ such that a population doubling would appear as an increase from 0 to 1.0 on the scale provided).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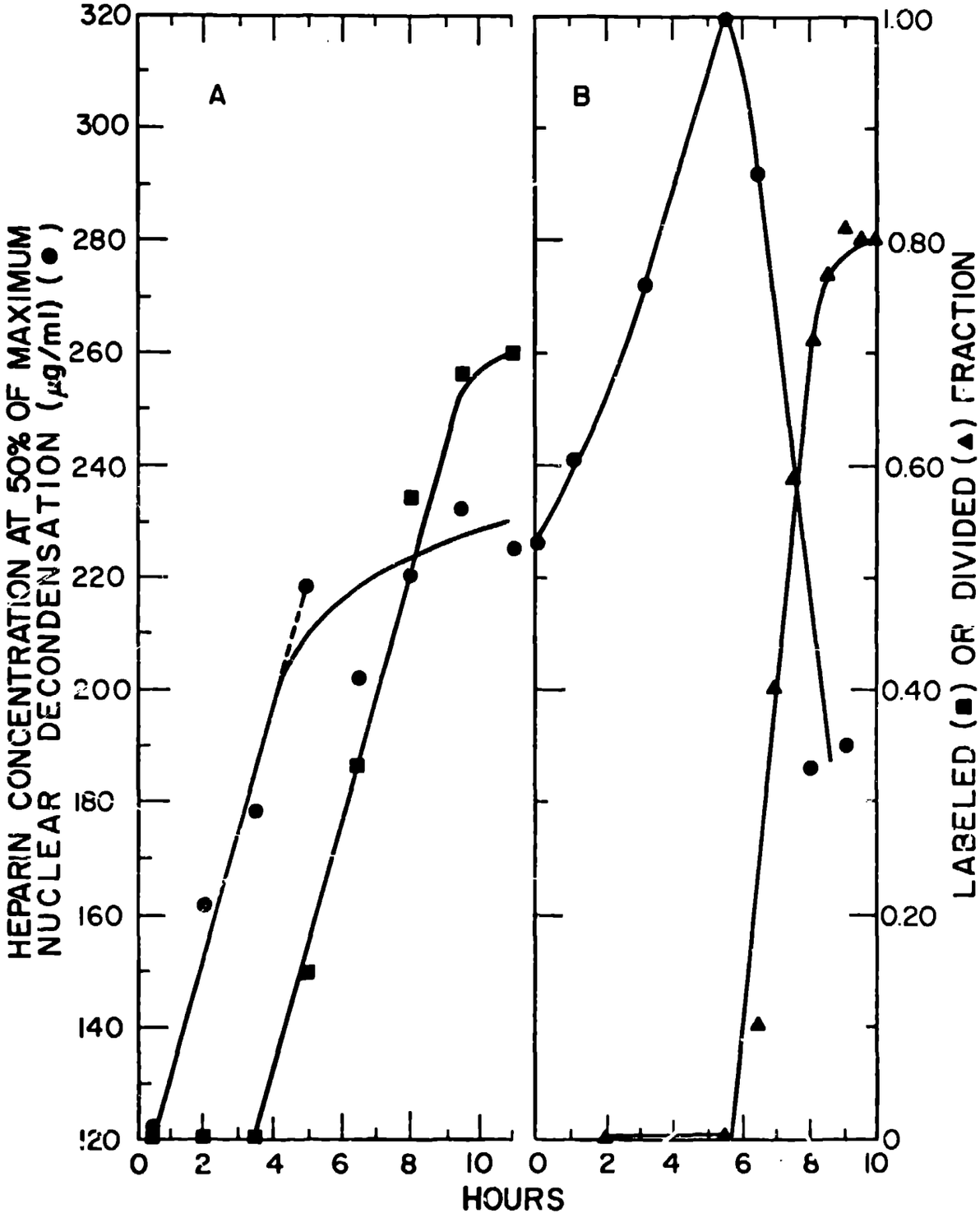
0
% DNA IN LIPID - PROTEIN COMPLEX (●)
LABELED (■) OR DIVIDED (▲) FRACTION

Figure 2. Heparin-mediated release of decondensed DNA from nuclei of synchronized CHO cells. A, Cells, prelabeled and synchronized as in Figure 1A, were resuspended in label-free medium and, at intervals thereafter, aliquots were removed, nuclei were isolated, and the concentration of heparin required to release 50% of the DNA was determined in the manner described previously (Hildebrand and Tobey, 1975). The ^{3}H-thymidine-labeled fraction was determined autoradiographically from aliquots taken from a non-prelabeled culture synchronized in parallel. B, Cells, prelabeled and synchronized as in Figure 1B, were washed and resuspended in fresh label-free medium. Aliquots were removed at intervals thereafter for determination of the cell number and concentration of heparin required to release 50% of the DNA from the nuclei. Symbols are as in Figure 1.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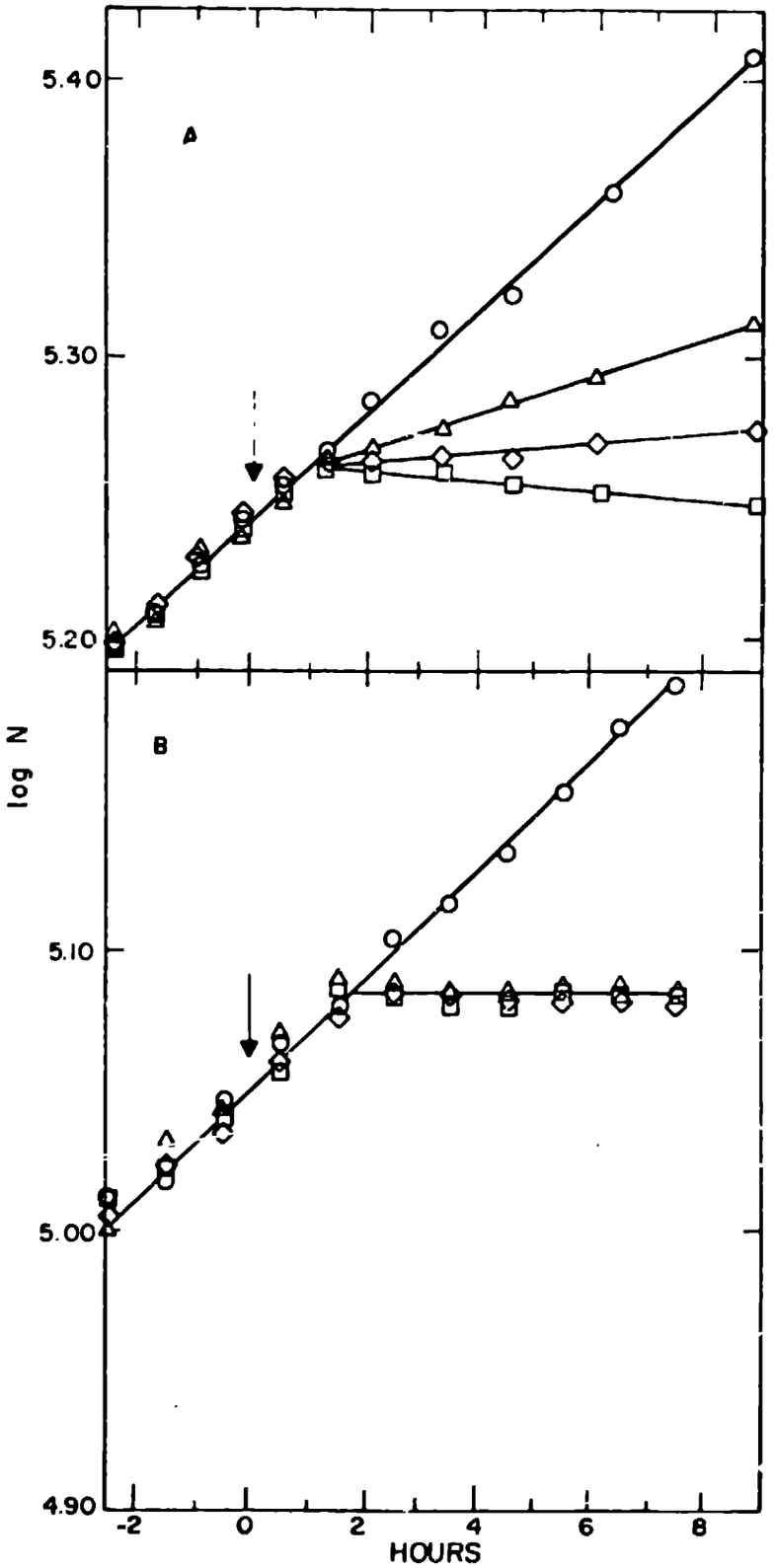
HEPARIN CONCENTRATION AT 50% OF MAXIMUM NUCLEAR DECONDENSATION (μg/ml) (●)
LABELED (■) OR DIVIDED (▲) FRACTION

Figure 3. Determination of the terminal point of action for neocarzinostatin and adriamycin in asynchronous cultures of CHO cells. A, An exponentially growing culture was split into four aliquots. At time 0, one culture received no drug and served as the control (circles). Neocarzinostatin was added to the other cultures, yielding final concentrations of 50 µg/ml (triangles), 400 µg/ml (diamonds), or 1,000 µg/ml (squares). B, Cultures of exponentially growing cells were treated with drug as above, yielding adriamycin concentrations of 0.5 µg/ml (diamonds), 1.0 µg/ml (squares), or 2.0 µg/ml (triangles). A fourth culture served as the drug-free control (circles). Cell number determinations were made with an electronic cell counter. For experimental details, see Tobey (1975).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HOURS

Figure 4. Drug-mediated arrest of progression at different stages of G_2 and mitosis in asynchronous cultures of CHO cells. The terminal point of action was determined for each agent, as in Figure 3. Drugs used were CLZ, chlorozotocin; STZ, streptozotocin; ADR, adriamycin; AMD, actinomycin D; MNU, 1-*trans*-(2-chloroethyl)-3-(4-methylcyclohexyl)-1-nitrosourea (Me-CCNU); BLM, bleomycin; CNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU); BNU, 1,3-*bis*(2-chloroethyl)-1-nitrosourea (BCNU); NCS, neocarzinostatin; CHM, cycloheximide; PUR, puromycin; CMD, Colcemid; ANA, antimycin A; DNP, 2,4-dinitrophenol; RTN, rotenone; and OLG, oligomycin. For details concerning G_2-inhibitory agents, see Tobey (1975) and for information concerning mitotic-inhibitory drugs, see Tobey *et al.* (1969).

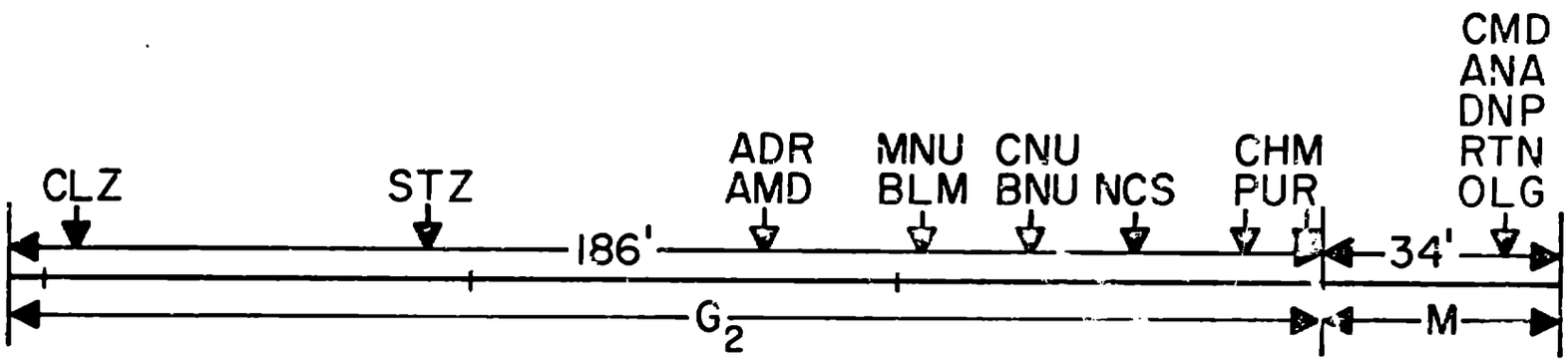
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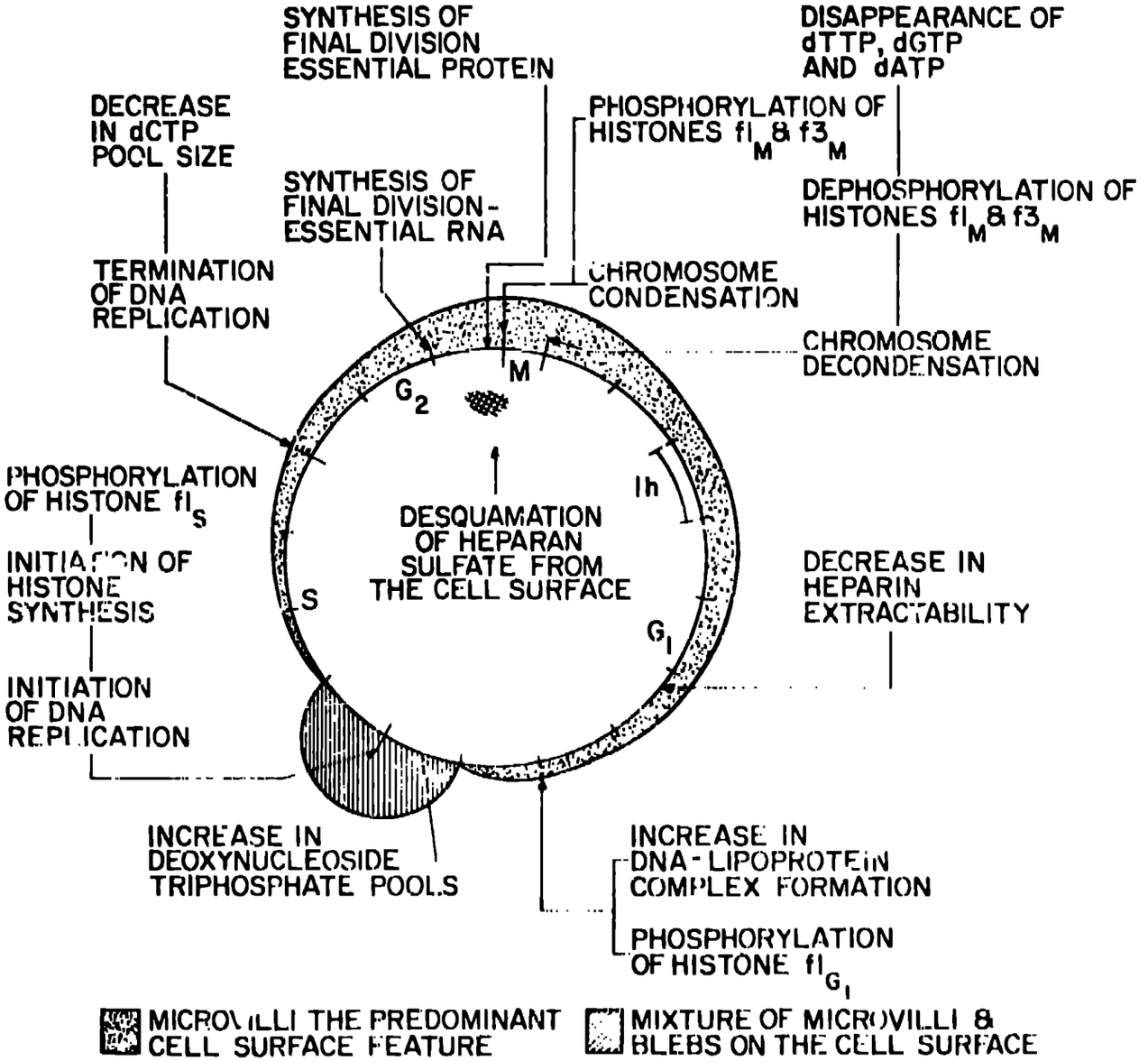
Figure 5. Temporal sequence of biochemical events in the CHO cell cycle. (Reprinted with permission from University of Alabama Press, Tobey et al. 1976.)


SYNTHESIS OF FINAL DIVISION ESSENTIAL PROTEIN
DISAPPEARANCE OF dTTP, dGTP AND dATP
DECREASE IN dCTP POOL SIZE
PHOSPHORYLATION OF HISTONES f1M & f3M
SYNTHESIS OF FINAL DIVISION-ESSENTIAL RNA
DEPHOSPHORYLATION OF HISTONES f1M & f3M
TERMINATION OF DNA REPLICATION
CHROMOSOME CONDENSATION
CHROMOSOME DECONDENSATION
M
G2
PHOSPHORYLATION OF HISTONE f1S
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DESQUAMATION OF HEPARAN SULFATE FROM THE CELL SURFACE
INITIATION OF HISTONE SYNTHESIS
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INCREASE IN DNA-LIPOPROTEIN COMPLEX FORMATION
PHOSPHORYLATION OF HISTONE f1G1
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